study by Jain et al. (17). However, that study did not include samples from extremely low birth weight infants.

It is well documented that atherosclerosis may originate during the foetal period and that both genetic and environmental factors can influence the cord blood lipid profile (18). Some experimental and clinical studies have shown the presence of fatty striae in the aorta during the foetal development period (19,20), while other epidemiological investigations have tried to link weight and lipid changes at birth to cardiovascular disease in adult life. However, the results of such studies remain inconclusive (21,22).

Table 2 Lipids and apolipoproteins in preterm infants soon after birth. Data are presented as the Mean, SD, Min and Max

	Mean	SD	Min	Max
TC (mg/dL)	85.0	24.8	14	133
TG (mg/dL)	26.9	18.0	8	66
HDLc (mg/dL)	29.0	8.6	5	41
LDLc (mg/dL)	35.5	15.4	5	98
apo A1 (mg/dL)	75.5	18.6	37	109
apo B (mg/dL)	27.2	10.1	8	60

Although the LDLc level in this study was negatively correlated with GA and BW, the HDLc level was positively correlated with BW and SD score for BW. It has previously been reported that foetal TC and LDLc levels decline from 30–33 to 42 weeks of GA (23,24). The decline in TC is attributed to the nearly 50% decrease in LDLc from 33 to 42 weeks of GA. Conversely, HDLc has been reported to remain constant from 30 to 42 weeks of GA (24). In human foetuses, increases in hepatic LDL receptor activity are positively correlated with gestational age and negatively correlated with LDLc concentration (25). Fujita et al. (26) also reported that LDLc concentration was negatively correlated with GA in cord blood, which may be partly explained by LDL receptor activity. The results of the present study indicate that prematurity affects LDLc level and poor foetal growth affects HDLc level at birth in premature infants.

In this study, apoA1 was positively correlated with BW, while apoB was negatively correlated with GA and BW. Furthermore, the apoB/apoA1 ratio was negatively correlated with GA, BW and SD score for BW. Donega et al. (27) also reported that the serum apoB level in cord blood is higher in preterm than in full-term newborns. Radunovic

Table 3 Correlations between lipids, lipoproteins, apolipoproteins and gestational age, birth weight and SD score for birth weight. The r values indicate Spearman's rank correlation coefficients

		TG	TC	HDLc	LDLc	apoA1	apoB
Gestational age	r	−0.17	−0.23*	0.12	−0.32**	0.22	−0.41**
Birth weight	r	−0.35**	−0.19	0.27*	−0.23**	0.23*	−0.40**
SD score for birth weight	r	−0.55**	−0.06	0.41**	−0.11	0.07	−0.07

*p < 0.05, **p < 0.01.

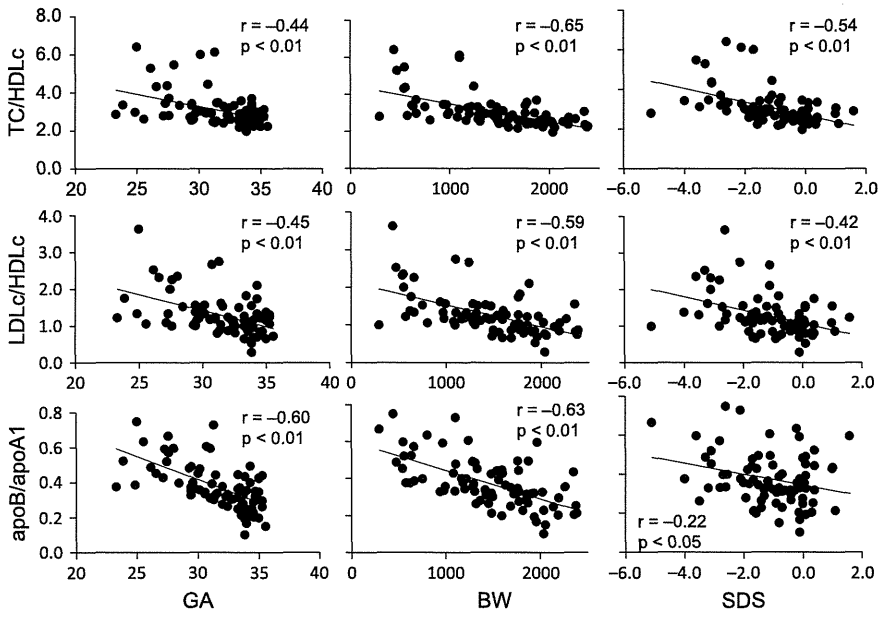

Figure 1 Relationship between TC/HDLc, LDLc/HDLc and apoB/apoA1 ratios and gestational age (GA), birth weight (BW) and SD score for birth weight (SDS).

et al. (28) previously demonstrated that apoB and apoB/apoA1 ratio were significantly higher in growth-restricted preterm foetus (mean GA of 28.4 weeks) than in normal growth foetus (mean GA of 26.9 weeks).

A limitation of the present work is that the influence of maternal lipid metabolism could not be eliminated, and the levels of maternal TC, TG, lipoprotein and apolipoprotein were not examined before delivery. Placental lipid transfer has been investigated in many studies. Maternal cholesterol can across the placenta (29) and cholesterol concentrations in maternal serum affect concentrations in neonates (30). However, this influence appears to be limited: foetuses can synthesise the cholesterol they need when their mothers have low circulating cholesterol (29).

In conclusion, the results of the present study suggest that prematurity and poor foetal growth may influence lipid and apolipoprotein metabolism and affect atherogenic indices at birth in preterm infants. Further research is needed to evaluate changes in the TC/HDLc, LDLc/HDLc and apoB/apoA1 ratios through adulthood as markers of the future development of cardiovascular disease.

CONFLICT OF INTEREST

The authors state that they have no conflicts of interest.

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Changes of lipoproteins in phenylalanine hydroxylase-deficient children during the first year of life

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ABSTRACT

Background: Influence of hyperphenylalaninemia on lipoproteins in early life remains unclear.
Methods: We enrolled 24 phenylalanine hydroxylase (PAH)-deficient children who were classified into a phenylketonuria (PKU) group ($n = 12$) lacking PAH activity and a benign hyperphenylalaninemia (HPA) group ($n = 12$) having partial PAH activity, and their 11 non-affected siblings. We measured serum total-cholesterol, low-density lipoprotein (LDL)-cholesterol, and high-density lipoprotein (HDL)-cholesterol levels together with apolipoproteins for the first year of life, and compared them with those of 30 age-matched healthy controls.
Results: The affected groups invariably had lower cholesterol levels than non-affected groups. At birth, HDL-cholesterol decrease was greatest and predominated over the LDL-cholesterol decrease: total cholesterol, 28/36% decrease to the control level in HPA/PKU; HDL-cholesterol, 33/51%; LDL-cholesterol, 20/28%. At 3 months, the opposite changes were observed: total cholesterol, 16/28%; HDL-cholesterol, 13/23%; LDL-cholesterol, 16/33%. At 12 months, LDL were still significantly lower in both groups (8/18%, $p < .05$ and .001), although HDL was significantly decreased only in the PKU group (15%, $p < .05$). Apolipoprotein A-I/A-II and B changed respectively in accordance with HDL-cholesterol and LDL-cholesterol changes. Despite similar phenylalanine levels, the PKU group invariably had lower cholesterol concentrations than the HPA group had.
Conclusion: Irrespective of phenylalanine concentrations, lipoprotein synthesis in PAH-deficient children, particularly in PKU children, was suppressed in early life.

1. Introduction

Phenylketonuria (PKU: OMIM, 261600) is an autosomal recessive disorder caused by hepatic phenylalanine hydroxylase (PAH; EC 1.14.16.1) deficiency. Adverse effects of hyperphenylalaninemia along with brain or neuron-related metabolic changes such as changed neurotransmitters have been increasingly documented [1–5].

Unless young PKU children receive phenylalanine-restricted diets, they develop convulsions, developmental delay, and mental retardation. However, the metabolic and nutritional effects of PKU and phenylalanine-restricted diets have been understood insufficiently to date.

Experimentally, suppressed cholesterol production in PKU has been suggested [6–8]. Clinically, Shulpis and his colleagues reported that PKU children adherent to phenylalanine-restricted diets with low cholesterol contents showed decreased low-density lipoprotein (LDL) cholesterol concentrations [9]. However, our recent studies for adult PKU demonstrated that, irrespective of diet or serum phenylalanine concentrations, total-cholesterol and LDL-cholesterol concentrations were, to some degree, decreased in them [10,11]. Changes

Abbreviations: PKU, phenylketonuria; PAH, phenylalanine hydroxylase.

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of cholesterol-derived metabolites functioning as bioactive substances such as oxysterols and vitamin D were also reported [10,11].

Considering that young children achieve rapid growth and development using various nutrients, including lipids, suppressed cholesterol production might be worrisome. However, reports of studies assessing the actual influence of PAH deficiency on lipoprotein metabolism in early life are rare.

In this study, to ascertain the influence of PAH deficiency on lipoprotein metabolism in early life, we performed chronological analyses of lipoprotein profiles in patients with PAH deficiency during the first year of life.

2. Subjects and methods

2.1. Subjects and sample collection

We enrolled 8 PKU patients (4 female, 4 male) at birth and 16 PKU patients (8 female, 8 male) after mass screening at around 5 days of age (Table 1). For the 24 affected children, the diagnoses of phenylalanine hydroxylase (PAH) deficiency were made by the analysis of dihydropteridine reductase activity in erythrocytes, biopterin loading test, and/or pteridine analysis in urine [1]. Patients were all found to have hyperphenylalaninemia by mass screening at around 5 days of age. They were classified into 2 groups (a PKU group comprising 12 children (female/male, 6/6) and a benign hyperphenylalaninemia (HPA) group comprising 12 children (female/male, 6/6) groups) according to the serum phenylalanine (phe) concentrations that had been determined repeatedly before the initiation of phenylalanine-restricted diets at the ages of 10–24 days. The PKU group included those children with serum fasting phenylalanine concentration of >600 μmol/l on normal formula or breast milk. The HPA group included the concentration of <600 μmol/l [1]. Soon after the diagnoses, the former patients strictly received restricted diets with phenylalanine-free milk, whereas the latter patients received mildly restricted diets. Consequently, both affected groups showed similar phenylalanine concentrations for the first years of life (Table 1).

In the same period, 11 non-PKU siblings (sibling group, 6 female and 5 male) and 30 healthy children (healthy control group, 16 female and 14 male) were enrolled. The data of the latter group were partially presented in our previous report [12].

2.2. Study design

For the affected patients and their non-affected siblings, we measured serum concentrations of total cholesterol, triglycerides (TG), LDL-cholesterol and high-density lipoprotein (HDL)-cholesterol together with serum phenylalanine concentration at birth, and at 3 months and 12 months of age. Simultaneously, we measured apolipoprotein (apo) A-I, A-II, B, and E. Their parameter concentrations at every age were compared with the respective controls' concentrations at the same ages. The time-to-time changes of parameters in each group were evaluated statistically.

For all groups, blood samples were obtained from cord veins at birth, or cubitus veins in the morning (at AM 7:30–8:00) before milk or meal intakes following 5–6 h and 7–9 h fasting, respectively, at the ages of 3–4 and 12 months. Serum lipid and apolipoprotein concentrations, and serum phenylalanine concentration were determined within 12 h of sample collection. The protocol was approved by the ethical committees of participating institutions. Written informed consent was obtained from the parents of all subjects.

2.3. Determination of serum lipids and apoproteins

Serum concentrations of TC and TG were determined using enzymatic methods with commercial kits (Kyowa Medex Co. Ltd.). Then HDL-C was measured using 13% polyethylene glycol (PEG 300; Wako Pure Chemical Industries Ltd.). LDL-C was measured using enzyme immunoassay with a commercial kit (LDL-C, Daiichi Pure Chemicals Co. Ltd.).

The Apo A-I, A-II, B, and E concentrations were measured using turbidimetric immunoassay with commercial kits (Daiichi).

Table 1
Changes in serum lipids and apolipoproteins during the first year of life.

	PKU ($n = 12$)	HPA ($n = 12$)	Non-affected siblings ($n = 11$)	Controls ($n = 30$)
M/F	6/6	6/6	6/5	14/16
Gestation pd. (week)	38–41	38–41	39–41	38–41
Birth weight (g)	2745–3210	2690–3150	2667–3333	2689–3465
Phe ranges (μmol/l)				
At birth	292 (30)[a,d,#]	123 (24)[a,##]	56 (15)	55 (17)
10–24 days	1352 (249)[a,d]	399 (97)[a]	75 (14)	73 (14)
3–4 M	189 (33)[a]	201 (46)[a]	69 (10)	63 (11)
12 M	211 (49)[a]	189 (38)[a]	72 (12)	66 (11)
TC (mg/dl)				
At birth	44 (3)[b,#]	50 (3)[##]	66 (8)	69 (14)
3–4 M	108 (14)[a,e]	127 (14)[b]	150 (13)	151 (20)
12 M	126 (17)[b,e]	138 (14)[c]	159 (14)	156 (23)
TC (mg/dl)				
At birth	44 (3)[b,#]	50 (3)[##]	66 (8)	69 (14)
3–4 M	108 (14)[a,e]	127 (14)[b]	150 (13)	151 (20)
12 M	126 (17)[b,e]	138 (14)[c]	159 (14)	156 (23)
TG (mg/dl)				
At birth	26 (6)[#]	29 (6)[##]	28 (9)	25 (22)
3–4 M	99 (9)[c]	97 (15)	91 (12)	88 (32)
12 M	95 (16)[c]	85 (9)	89 (20)	80 (29)
LDL-C (mg/dl)				
At birth	18 (2)[c,#]	20 (3)[##]	26 (6)	25 (8)
3–4 M	49 (11)[a,e]	61 (11)[c]	73 (10)	73 (16)
12 M	62 (13)[a,e]	70 (11)[c]	78 (10)	76 (19)
HDL-C (mg/dl)				
At birth	19 (2)[a,#]	26 (3)[c,##]	36 (8)	39 (13)
3–4 M	41 (5)[b,f]	46 (8)[c]	53 (5)	53 (9)
12 M	45 (4)[b,f]	51 (5)	54 (5)	53 (10)
Apo A-I (mg/dl)				
At birth	44 (3)[a,#]	59 (4)[c,##]	84 (10)	89 (15)
3–4 M	93 (8)[b,f]	109 (17)[c]	117 (9)	120 (21)
12 M	106 (11)[c]	112 (11)	120 (7)	119 (22)
Apo A-II (mg/dl)				
At birth	8 (4)[a,#]	12 (4)[c,##]	18 (4)	20 (3)
3–4 M	20 (3)[b,f]	23 (5)[c]	24 (6)	25 (5)
12 M	22 (2)[c]	24 (4)	25 (3)	25 (7)
Apo B (mg/dl)				
At birth	14 (1)[c,#]	17 (1)[##]	20 (5)	19 (5)
3–4 M	51 (9)[a,e]	64 (10)[b]	74 (11)	75 (17)
12 M	63 (14)[a,f]	72 (11)[c]	79 (11)	78 (19)
Apo E (mg/dl)				
At birth	3.1 (0.5)[c,#]	3.6 (0.4)[##]	4.4 (0.7)	4.9 (1.5)
3–4 M	3.9 (0.6)[c]	4.3 (0.9)[c]	5.0 (0.7)	4.8 (1.2)
12 M	4.3 (0.7)[c]	4.4 (0.9)	4.7 (0.9)	4.7 (1.6)

Data of lipids and apolipoproteins are presented as mean (SE).
PKU, phenylketonuria; HPA, benign hyperphenylalaninemia; Non-affected Sib, healthy sibling; Phe, serum phenylalanine: TC, total cholesterol; TG, triglycerides; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.
[#] Blood samples were collected from 4 of the 12 PKU patients.
[##] Blood samples were collected from 4 of the 12 HPA patients.
[a] $p < .001$ vs. age-matched controls.
[b] $p < .01$ vs. age-matched controls.
[c] $p < .05$ vs. age-matched controls.
[d] $p < .001$ vs. HPA patients.
[e] $p < .01$ vs. HPA patients.
[f] $p < .05$ vs. HPA patients.

2.3.1. Statistic analyses

Differences between the 2 groups were estimated at birth, at 3 months and at 12 months of age using 2-sided Student *t*-tests. For each group, the time-to-time changes of parameter concentrations were estimated repeatedly using ANOVA tests. Any p value < .05 was regarded as significant.

3. Results

3.1. Serum phenylalanine concentrations in affected groups and non-affected groups

At birth, we were able to determine serum phenylalanine concentrations for 4 of 12 PKU children, 4 of 12 HPA children and 11 non-affected sibling children, and compared them with those of 30 healthy control children (Table 1). Phenylalanine concentrations of the affected children, particularly the PKU children, were overtly high compared to those of non-affected siblings and the control children. Of the 24 affected children, 16 were enrolled after mass screening at around 5 days of age, although the 30 healthy control members never dropped out during this study.

At 10–19 days of age when both the 24 affected children took breast milk or normal formula containing phenylalanine, as did 11 non-affected siblings and 30 control children, the 12 PKU children exhibited extremely high phenylalanine concentrations greatly in excess of 600 μmol/l. 12 HPA children also exhibited high phenylalanine concentrations, but the values never exceeded 600 μmol/l (Table 1).

Thereafter, the affected children adhered to phenylalanine-restricted diets. Most of them achieved the recommended phenylalanine concentrations. However, those concentrations were still higher than those of non-affected children (Table 1).

3.2. Serum lipid concentrations in affected groups and non-affected groups

At birth, total-cholesterol and HDL-cholesterol concentrations in the affected group were decreased greatly compared to those of the control group. The percentage values of lipid decreases to the control group's concentrations were the following: total-cholesterol, 28 (HPA group)–36% (PKU group); HDL-cholesterol, 49–67%. Consequently, HDL-cholesterol in the PKU group was much lower than those of non-affected children (p < .001 vs. healthy controls). The Apo A-I and A-II concentrations were also greatly decreased in the affected children, as was the HDL-cholesterol concentration (p < .001 vs. healthy controls). The decreases of LDL-cholesterol and apo B concentrations were also considerably less in PKU children (about 28% decrease, p < .05), but such decreases were not statistically significant for HPA children (about 20% decrease, p > .05). In addition, apo E concentrations were decreased in PKU children (about 40% decrease, p < .05) and HPA children (about 30% decrease, p < .05).

At 3 months, the HDL-cholesterol, apo A-I and A-II in the affected 2 groups were increased about 2-fold. The changes were greater (p < .001) than those in the non-affected 2 groups, which showed about a 1.5-fold increase (p < .01) (Table 1 and Fig. 1). The LDL-cholesterol and apo B concentrations were 2.5-fold to 3-fold increased in all groups. HDL-cholesterol, apo A-I and A-II concentrations in the affected 2 groups were still significantly lower than those in the non-affected children: PKU group, p < .01 vs. healthy control group; HPA group, p < .05. LDL-cholesterol and apo B concentrations in the affected groups remained considerably lower than those in non-affected 2 groups (PKU group, p < .001; HPA group, p < .01). Furthermore, apo E concentrations in the affected groups were significantly lower than those in non-affected groups (p < .05). In contrast, the TG concentration was not different between these four groups.

At 12 months, increases of LDL-cholesterol and apo B in the affected groups were greater than those in the non-affected groups showing no significant changes. Such changes were significant (p < .01 for PKU group and p < .05 for HPA group) (Table 1 and Fig. 1). Increases of HDL-cholesterol and apo As in the affected groups were also significant (p < .05), although such significant increases were not observed in non-affected groups. Consequently, differences in lipid and apo concentrations between the affected and non-affected groups became smaller. Nevertheless, total cholesterol and LDL-cholesterol concentrations, and apo B concentrations in the affected were still lower than non-affected groups: PKU group, p < .01 for total-cholesterol concentration and p < .001 for LDL-cholesterol and apo B concentrations: HPA group, p < .05 for total-cholesterol and LDL-cholesterol concentrations and apo B concentration. The PKU group, but not the HPA group showed significantly lower HDL-cholesterol, apo A-I and A-II concentrations: p < .01 for HDL-cholesterol concentration and p < .05 for apo A-I and A-II concentrations. Apo E concentration remained low in the PKU group, but not the HPA group.

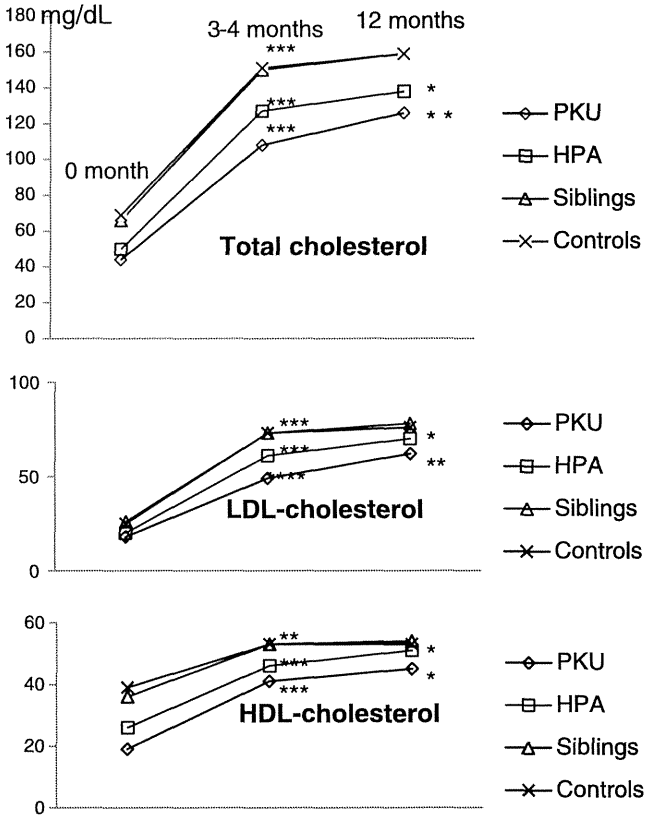

Fig. 1. Lipid changes in respective groups during the observation period. PKU, phenylketonuria; HPA, benign hyperphenylalaninemia, LDL-cholesterol, low-density lipoprotein cholesterol; HDL-cholesterol, high-density lipoprotein cholesterol. *p < .05, **p < .01, ***p < .001 vs. pre-values.

4. Discussion

We performed chronological analyses for lipoprotein profiles in children with PAH deficiency for the first year of life. This report is the first describing details of lipoprotein metabolism in PKU patients of early life.

Greater influences of PAH on HDL-cholesterol and LDL-cholesterol concentrations in early life than in later life were found. The results also show that the magnitude of the influence on lipoprotein metabolism differs by the clinical phenotypes, but not by the serum phenylalanine concentration. The change of lipoprotein profile in the PKU group lacking PAH activity was always significantly greater than that in the HPA group having partial PAH activity, even though their phenylalanine concentrations were mutually comparable.

Repeated comparisons of lipoprotein profiles between affected and non-affected groups revealed that the influence of PAH deficiency on the lipoprotein metabolism changes during the first year of life: At birth, the decreases of HDL-cholesterol and the 2 major apolipoproteins on HDL particles, apo A-I and apo A-II, predominated over the decreases of LDL-cholesterol and apo B on LDL particles. In contrast, at 3 months, the decreases of LDL-cholesterol and apo B predominated over the decreases of HDL-cholesterol, apo A-I and apo A-II concentrations. At 12 months, decreases of LDL-cholesterol and apo B were decreased to some degree in the 2 affected groups, whereas HDL-cholesterol and apo A-I/A-II were decreased significantly only in PKU groups. Consequently, suppression patterns of lipids were not consistent during the first year of life.

In PKU, cholesterol production and production of cholesterol-derived metabolites such as oxysterols and vitamin D have been suggested to be suppressed [6–11]. Our recent study also showed that total-cholesterol and LDL-cholesterol concentrations were decreased about 10% compared to the respective control concentrations, irrespective of the serum phenylalanine concentration, in PKU adults [10,11]. In contrast, the decrease of HDL-cholesterol was minute in them. Decrease of LDL-cholesterol at 12 months of age in the PKU group was comparable to that in adult PKU, but no decrease of HDL-cholesterol in the PKU group was observed in PKU adults.

As mechanisms of suppressed cholesterol production in PKU, reduced activities of 2 key enzymes for cholesterol production, 3-hydroxy-3methylglutaryl-CoA reductase and mevalonate-5-pyrophosphate decarboxylase have been suggested [6–8]. Our recent study showed that oxysterols reflecting cholesterol production – lanosterol and lathosterol – were decreased in adult PKU [11]. In contrast, campesterol and sitosterol as markers for cholesterol absorption from the intestine remained unchanged in them. If this is a case in neonate and infant with PAH deficiency, their liver PAH activity but not serum phenylalanine concentration might be deeply associated with the suppression of cholesterol production.

The lipoprotein profile in fetuses differs greatly from that in adults for the reason that lipoprotein synthesis is low because of immature hepatic function and the absence of intestinal lipid absorption [12,13]. The major plasma lipoprotein in fetuses is HDL, whereas that in adults is LDL. Furthermore, HDL particles in the fetus contain much apo E. Lipoprotein metabolism changes drastically for the first year after birth. In this context, it is plausible that the impact of PAH deficiency on lipoprotein metabolism differs according to age. This study showed that the apo E concentration was, to some degree, decreased in the affected groups, in particular at birth. We were able to infer that this decrease of apo E was, to considerable degrees, attributable to the suppressed HDL synthesis.

Consequently, substantial and considerable decreases of lipoprotein production in PAH deficiency of early life were demonstrated. Nevertheless, the actual shortcomings of the lipoprotein production suppression in neonates and infants remain to be elucidated. Cholesterol is transformed to bioactive oxysterols and vitamin D [14–22]. Moreover, crucial roles of brain-related oxysterols such as 24S-hydroxycholesterol in the brain have been suggested [16–18].

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Serum myeloperoxidase/paraoxonase 1 ratio as potential indicator of dysfunctional high-density lipoprotein and risk stratification in coronary artery disease

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ABSTRACT

Objective: Granular leukocyte-derived myeloperoxidase (MPO) promotes oxidation of lipoproteins, while paraoxonase 1 (PON1) has antioxidant properties for high-density lipoprotein (HDL). We evaluated their effects on coronary risk stratification and function of lipoproteins.
Methods and results: A total 158 patients who had previously undergone percutaneous coronary intervention and who had been hospitalized for coronary re-angiography were enrolled. Coronary lesions (restenosis or de novo lesion) were observed in 84 patients but not associated with conventional lipid profile. In contrast, serum MPO levels and PON1 activities were significantly associated with the prevalence of coronary lesions. The high MPO/PON1 ratio, when cutoff values were set at 1.59, was independently correlated with restenosis (odds ratio 6.4, 95% CI 2.2–19.3, $P = 0.001$) and de novo lesions (odds ratio 3.5, 95% CI 1.3–9.4, $P = 0.014$). We isolated HDL from patients with high or low MPO/PON1 ratio, and compared anti-inflammatory properties of HDL. Human umbilical vein endothelial cells were stimulated with inflammatory cytokine, and the expression of vascular cell adhesion molecule-1 (VCAM-1) was evaluated. HDL isolated from patients with low serum MPO/PON1 ratio inhibited VCAM-1 expression significantly greater than that with high MPO/PON1 ratio. We also demonstrated that the cholesterol efflux capacity of apolipoprotein B-depleted serum from patients with high MPO/PON1 ratio was significantly decreased than that with low MPO/PON1 ratio.
Conclusions: MPO/PON1 ratio could be a useful marker for secondary prevention of coronary artery disease through modulation of HDL function.

1. Introduction

Although high plasma level of low-density lipoprotein cholesterol (LDL-C) is an established risk factor for coronary artery diseases (CAD), the adequate LDL-lowering therapy with statins has been shown to reduce the prevalence of CAD by 30–40% at most [1,2]. LDL displays several phenotypes such as oxidized LDL and small dense LDL, which accelerate atherosclerosis much greater than native LDL, therefore the modulation of LDL size and subclasses may be a next therapeutic target for the residual cardiovascular risk [3]. On the other hand, high-density lipoprotein cholesterol (HDL-C) is a negative risk factor for CAD [4]. HDL exhibits a variety of anti-atherogenic functions including anti-inflammatory and anti-oxidative as well as promoting reverse cholesterol transport [5]. However, it has been reported that HDL may lose its anti-atherogenic properties and become pro-atherogenic (dysfunctional) under conditions such as inflammation, diabetes, and oxidative stress [5]. These lines of evidence suggest that the function of lipoproteins may significantly modulate and predict the progression of CAD in addition to the quantity of lipoproteins.

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Myeloperoxidase (MPO) is a heme peroxidase, which is produced from granular leukocytes. Besides its antibacterial effects, MPO causes oxidative- or chemical-modification against circulating lipoproteins [6]. In addition, MPO generates reactive oxygen and nitrogen species which facilitate lipid peroxidation, protein nitration, and protein carbamylation [6]. LDL which undergoes these modifications promotes foam cell formation. On the other hand, the modified HDL loses its cholesterol efflux activity and anti-inflammatory properties [7]. Through these functional changes, MPO may promote the progression of atherosclerosis.

Paraoxonase 1 (PON1) is one of the major HDL-associated proteins. PON1 is strongly lipophilic and co-exists with apolipoprotein (apo)A-I and apo-J in HDL particles. PON1 hydrolyzes organophosphates and its activity is stabilized in the presence of the apolipoprotein [8]. PON1 inhibits oxidation of LDL, and reduce oxidative stress in blood vessels [8]. In fact, previous studies have shown that the activities of PON1 are negatively associated with the prevalence of cardiovascular events [9]. Given the function of lipoproteins is impaired by MPO and improved by PON1, the plasma level or activity of MPO and PON1 is a marker and modulator of not only lipoprotein functions but also initiation and progression of CAD. However, it has not been fully elucidated whether these molecules, alone or in combination, predict the prevalence of CAD. In the present study, therefore, we evaluated serum MPO level and PON1 activity in patients with chronic CAD, and validated a hypothesis that the MPO/PON1 ratio could be a useful marker for secondary prevention of CAD through modulation of HDL functions.

2. Materials and methods

2.1. Study subjects

From April 2008 to March 2010, we enrolled 158 consecutive patients who had previously undergone successful percutaneous coronary intervention (PCI) with stenting one or more times and who had been admitted to Kobe University Hospital for the purpose of coronary angiography (CAG) because of 6-month follow-up or stable angina or inducible ischemia (CAD group). On the other hand, 174 patients without past history of CAD were enrolled as a non-CAD group in the same period. In addition, the CAD group was classified according to the following definition: patients who showed restenosis in the original stented segment (in-stent restenosis; ISR group); patients with occurrence of other non-target coronary atherosclerotic lesions (de novo lesion group); and patients with neither ISR nor de novo lesion (no lesion group). ISR and de novo lesions were defined as displaying luminal stenosis ≥75% and demonstrating ischemia in the perfusion area of narrowed coronary by stress myocardial scintigram. Weight and height were measured and body mass index (BMI) was obtained by dividing a patient's weight by their height squared. Patients who had acute coronary syndrome, renal failure (creatinine >3.0 mg/dL), and a history of cancer in the previous 5 years were excluded. To exclude inflammatory diseases (e.g., infections, malignancies, autoimmune diseases), patients whose serum C-reactive protein (CRP) was more than 1.0 mg/dL were also not assigned because MPO is produced from granular leukocytes, and serum MPO levels might be influenced by inflammatory conditions. All patients gave written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. The investigation conforms to the principles outlined in the Declaration of Helsinki.

2.2. Blood chemistry

Serum was obtained after overnight fast. LDL-C, HDL-C, apoA-I, apoA-II, apoB, apoC-II, apoC-III, apoE, glucose, hemoglobin (Hb) A1c, high sensitivity C-reactive protein (hsCRP), and triglyceride (TG) were measured using standard methods at Kobe University Hospital. PON1 paraoxonase activity was measured using paraoxon as the substrate by SRL (Kobe, Japan). The rate of generation of p-nitrophenol was determined spectrophotometrically. Residual serum was stored at −80 °C for assays of MPO and PON1 arylesterase activity. Arylesterase activity was measured as follows; Serum was preincubated with 10 μM eserine (Sigma, St. Louis, MO, USA) for 10 min at room temperature for the inhibition of butyrylcholinesterase activity. After adding 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and 1 mM phenyl acetate (Wako Chemicals, Richmond, VA, USA), the rate of hydrolysis of phenyl acetate was determined spectrophotometrically at 270 nm in an automated Shimadzu UV-1600, UV-visible Spectrophotometer (Shimadzu, Kyoto, Japan) [10]. Serum MPO levels were measured by human MPO ELISA kit (Hycult Biotechnology, Uden, Netherland) as per manufacturer's protocol.

2.3. Inhibition of tumor necrosis factor-α induced vascular cell adhesion molecule-1 expression by HDL

HDL fraction was obtained from the serum with MPO/PON1 ratio <1 or >3 by ultracentrifugation as described elsewhere [11]. Human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (Manassas, VA, USA). Subconfluent HUVECs were seeded in a 24 well-plate at 1.2×10^5 cells/well. After 16 h, the media was replaced with new one containing human HDL (100 μg protein/well) which was purified from serum with MPO/PON1 ratio <1 or >3, or bovine serum albumin (control, 100 μg protein/well). HUVECs were incubated for 16 h, then stimulated with or without tumor necrosis factor (TNF)-α (10 ng/ml) for 6 h. Experiments were terminated by aspiration of the medium and washing cells twice with 500 μl of cold phosphate buffered saline. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by cDNA preparation using reverse transcript agent, then quantitative real-time polymerase chain reaction (PCR) was performed. PCR primers for human vascular cell adhesion molecule-1 (VCAM-1) and glyceraldehyde 3-phosphate dehydrogenase were purchased from Takara-Bio Perfect Real Time Support System (Takara, Shiga, Japan).

2.4. Cholesterol efflux assays

To quantify HDL efflux capacity, apolipoprotein B-depleted serum was prepared from patients with MPO/PON1 ratio >3 or <1 as previously reported [12]. THP-1 human monocyte cells were seeded into 12 well plates at density of 3×10^6 cells per well. Cells were differentiated into macrophages with phorbol 12-myristate 13-acetate (Sigma, final concentration 100 ng/ml) for 60 h. Cellular cholesterol was labeled with 1 μCi of [^{3}H]-cholesterol for 12 h. Then cells were washed with PBS and incubated for 18 h with medium containing 0.2% BSA and TO-901317 (Cayman, final concentration 10 μM/l) to up-regulate ABC transporters. Cells were washed again with PBS and incubated for 4 h with medium containing 2% apolipoproteinB-depleted serum. After 4 h, medium was collected and centrifuged at 15,000 rpm for 5 min and then aliquots of supernatant were counted in liquid scintillation. The cell layer was washed twice with PBS and incubated with isopropanol for 1 h at room temperature, and radioactivity was measured as for the supernatant. Cholesterol efflux was given as the following; the proportion of [^{3}H]-cholesterol counts in the medium to the total

counts present on the well (i.e., sum of medium and cells) were calculated, and then background values (i.e., the efflux in medium without apolipoprotein B-depleted serum) were subtracted from the respective experimental values. All samples were analyzed in triplicate.

2.5. Statistical analysis

All statistical analyses were performed using Stata10 (College Station, TX, USA). All data are expressed as mean ± s.d., unless otherwise specified. *P* value for two groups was determined by Student's *t*-test or the Mann–Whitney test according to the data distribution, with or without normality. The χ^2-test was used to compare categorical variables between the groups. The differences among multiple groups were analyzed by Kruskal–Wallis test. Multiple regression analyses were performed to evaluate the relationship between the prevalence of coronary lesions (ISR, de novo lesions, or both) in the CAD group and other clinical parameters. *P* value <0.05 was considered significant. The receiver operating characteristic curve (ROC curve) was used to determine MPO/PON1 ratio cut-off point. In order to investigate if MPO/PON1 ratio provide more effective CAD risk stratification than serum MPO levels alone, we calculated net reclassification improvement (NRI) and integrated discrimination improvement (IDI) according to Pencina et al. [13]. The associations of MPO and PON1 with other clinical parameters were estimated using linear regression analysis.

3. Results

3.1. Conventional risk markers do not associate with the prevalence of coronary lesions in patients with a past history of CAD

In 158 patients who underwent CAG re-examination, 44 patients showed ISR, 40 patients had de novo lesions, and 74 patients did not show either ISR or de novo lesions. There were no significant differences in the baseline characteristics, including what types of medications they were prescribed, among those groups (Table 1). Original stent types did not vary between the ISR group and the no lesion group as shown in Table 1. The percentage of patients with diabetes mellitus seemed higher in the de novo lesion group but no statistical significance was found.

Table 1
Baseline characteristics of the CAD patients.

	No lesion ($n = 74$)	De novo ($n = 40$)	ISR ($n = 44$)	*P*-value
Age (years)	67.4 ± 10.0	68.5 ± 10.9	67.1 ± 9.3	0.693
Sex (Male %)	74.2	84.9	89.1	0.059
Family history (%)	25.6	26.0	33.3	0.605
Current smoking (%)	28.6	23.1	18.5	0.384
Hypertension (%)	89.1	83.0	80.0	0.292
Diabetes mellitus (%)	44.6	57.7	57.4	0.191
Dyslipidemia (%)	80.2	76.9	79.6	0.893
Waist (cm)	88.5 ± 9.2	88.3 ± 8.9	88.7 ± 9.8	0.964
BMI (kg m^{-2})	24.6 ± 3.1	24.4 ± 3.7	24.8 ± 4.2	0.857
SBP (mmHg)	126.2 ± 16.0	127.0 ± 15.3	126.9 ± 16.0	0.923
DBP (mmHg)	66.3 ± 9.4	65.8 ± 10.2	65.7 ± 9.6	0.850
Stent Types (%)				0.484
BMS	27.5	13.9	19.6	
DES	60.0	66.7	67.4	
Both	12.5	19.4	13.0	
Medications (%)				
Aspirin	90.3	94.3	96.4	0.3456
Thienopyridine	62.4	58.5	78.2	0.654
Statin	73.1	66.0	61.8	0.338
ACEI/ARB	63.4	66.0	56.4	0.553

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMS, bare-metal stent; DES, drug-eluting stent; ISR, in-stent restenosis. Values are the mean ± s.d. *P* values were obtained using the Kruskal–Wallis test for continuous variables and the χ^2-test for categorical variables.

The LDL-C level of patients with a past history of CAD was lowered to the target level (<100 mg/dL) in accordance with the guideline of the Japan Atherosclerosis Society for secondary prevention of CAD, while HDL-C levels in the CAD group were still lower than those of the non-CAD group (LDL-C, 95.7 ± 23.8 vs. 114.1 ± 31.8, $p < 0.001$; HDL-C, 47.2 ± 12.1 vs. 55.6 ± 16.0, $p < 0.001$, respectively). On the other hand, in the CAD group, serum lipid parameters were not related to whether restenosis or de novo lesions were present, indicating that quantitative changes in lipid profile are not sufficient to predict a recurrence of CAD (Table 2). Levels of hsCRP also had no relationship with the presence or absence of coronary lesions in the CAD group. On the other hand, although not statistically significant, HbA1c tended to be larger in the former than in latter (Table 2).

3.2. Serum MPO/PON1 ratio is an independent risk indicator of the recurrence of coronary artery disease

We next focused on MPO and PON1 that potentially represent functional qualities of plasma lipids and lipoproteins. First, we compared those markers between patients with and without a past history of CAD. There was no significant difference in serum MPO levels between CAD and non-CAD groups (207.1 (158.7–255.5) ng/mL vs. 237.8 (202.6–273.0) ng/mL, $p = 0.324$), whereas PON1 activity as paraoxonase was significantly lower in the CAD group than in the non-CAD group (220.0 (209.7–230.3) U/L vs. 251.1 (237.5–264.8) U/L, $p < 0.001$). Linear regression analysis demonstrated weak but significant inverse correlations of PON1 paraoxonase activity with age ($r^2 = 0.085$, $p < 0.001$) and the rate of concurrent diabetes mellitus ($r^2 = 0.049$, $p < 0.001$). Age and the percentage of patients with diabetes mellitus at baseline differed significantly between the non-CAD and CAD groups (age, 60.2 ± 13.9 years vs. 67.6 ± 10.0 years, $P < 0.001$; diabetes mellitus, 15.7% vs 51.5%, $P < 0.001$, respectively), indicating that those factors might affect the decrease in PON1 paraoxonase activity in CAD patients.

As shown in Table 2, Kruskal–Wallis test indicated significant differences of serum MPO mass and PON1 paraoxonase activity among CAD patients depending on whether they had coronary lesion at CAG re-examination. We also evaluated arylesterase activity to elucidate whether PON1 enzyme activity is independent of paraoxonase polymorphisms. Significant differences in arylesterase activity among no lesion, de novo lesion, and ISR groups were observed as well (101.0 ± 21.5 U/L vs. 87.0 ± 15.0 U/L vs. 87.1 ± 16.2 U/L, respectively, $P = 0.006$).

When we evaluated the relative effect of these parameters by calculating serum MPO mass/PON1 paraoxonase activity (MPO/PON1 ratio), there was no significant difference in MPO/PON1 ratio between the non-CAD and CAD groups (1.11 (0.93–1.30) vs. 1.20 (0.80–1.60), $p = 0.731$). In contrast, MPO/PON1 ratio was significantly different among CAD patients depending on the recurrence of coronary lesion (Table 2). We investigated if co-assessment of PON1 improves the diagnostic accuracy of MPO in CAD risk stratification by assessing NRI and IDI. The category-free NRI of MPO/PON1 ratio for CAD risk stratification was greater than that of MPO alone (39.3% vs 34.7%, $p = 0.023$ and 0.040, respectively). The IDI after addition of MPO/PON1 ratio to the established risk factors (gender, age, smoking, hypertension, diabetes mellitus and dyslipidemia) was also large compared with that of MPO alone (10.4% vs. 8.8%, $p < 0.001$ in both). In multivariate logistic regression analysis that adjusted for age, gender, history of smoking, hypertension,

Table 2
Comparisons of laboratory parameters among the CAD patients.

	No lesion	De novo	ISR	*P*-value
	(*n* = 74)	(*n* = 40)	(*n* = 44)	
T-Cho, mg/dL	163.9 ± 28.3	166.0 ± 29.6	167.9 ± 30.3	0.787
LDL-C, mg/dL	94.4 ± 24.5	96.0 ± 25.1	97.5 ± 21.4	0.689
HDL-C, mg/dL	47.0 ± 12.4	46.5 ± 10.5	48.4 ± 13.2	0.532
TG, mg/dL	133.7 ± 69.7	132.3 ± 71.8	129.2 ± 69.4	0.886
non HDL, mg/dL	117.0 ± 27.2	119.5 ± 28.2	119.5 ± 27.4	0.894
apo-AI, mg/dL	131.5 ± 23.9	130.8 ± 20.7	130.6 ± 23.0	0.990
apo-AII, mg/dL	32.5 ± 6.4	31.3 ± 5.8	31.5 ± 6.3	0.371
apo-B, mg/dL	72.4 ± 14.8	73.3 ± 16.2	73.7 ± 14.4	0.920
apo-CII, mg/dL	4.2 ± 1.5	4.3 ± 1.8	4.5 ± 2.0	0.605
apo-CIII, mg/dL	10.6 ± 3.8	10.7 ± 4.3	10.8 ± 3.8	0.908
apo-E, mg/dL	3.9 ± 1.0	3.8 ± 1.0	3.8 ± 1.0	0.489
Glu, mg/dL	103.4 ± 27.6	111.7 ± 36.0	107.8 ± 32.3	0.465
HbA1c, %	5.8 ± 0.7	6.3 ± 1.4	6.3 ± 1.3	0.075
hsCRP, mg/dL	0.14 ± 0.22	0.12 ± 0.14	0.10 ± 0.12	0.639
MPO mass, ng/mL	123.6 (101.2–145.9)	266.0 (134.1–397.8)	294.0 (174.8–413.2)	0.021
PON1 activity, U/L	234.3 (219.4–249.3)	200.6 (182.9–218.4)	213.6 (191.3–235.8)	0.009
MPO/PON1 ratio	0.56 (0.46–0.66)	1.79 (0.67–2.91)	1.77 (0.73–2.81)	0.002

Abbreviations: ISR, in-stent restenosis; T-Cho, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; apo, apolipoprotein; Glu, glucose; Hb, hemoglobin; hsCRP, high sensitivity C-reactive protein; MPO, myeloperoxidase; PON, paraoxonase. Values are the mean ± s.d. or geometric mean (95% confidence interval).
P values were obtained using Kruskal–Wallis test.

dyslipidemia, and diabetes mellitus, elevated MPO/PON1 ratio was associated with the prevalence of coronary lesion (ISR or de novo lesion) in the CAD group (OR 2.89, 95% CI 1.36–6.12, $P = 0.006$). The prognostic cutoff for MPO/PON1 ratio was set according to the ROC curve at 1.59 (see Supplementary Appendix), with sensitivity, specificity, and overall accuracy of 25.4%, 98.6%, and 64.4%, respectively. The area under the curve was 0.662 (95% CI 0.570–0.753, $p < 0.001$). Logistic regression analysis, adjusted for the factors indicated above, revealed that MPO/PON1 ratio >1.59 was independently associated with incidence of both ISR and de novo lesion as shown in Table 3.

3.3. MPO/PON1 ratio represents anti-inflammatory function and cholesterol efflux capacity of HDL

Meanwhile, we also investigated whether serum MPO/PON1 ratio is associated with the HDL properties using ex vivo systems. First, a relationship between MPO/PON1 ratio and anti-inflammatory function of HDL was examined. Complementary cell culture experiments revealed that TNFα markedly increased VCAM-1 expression in HUVEC, and the cytokine-induced VCAM-1 expression was significantly attenuated by pretreatment of the cell with HDL from CAD patients (Fig. 2A). Interestingly, the inhibitory effect of HDL was greater with low MPO/PON1 ratio than that with high MPO/PON1 ratio (Fig. 1A). Such relationships were held among non-CAD patients (Fig. 1B).

Next, to assess the impact of MPO/PON1 ratio on cholesterol efflux from macrophage, ex vivo efflux experiments were performed. Cholesterol efflux capacity of apolipoprotein B-depleted serum was reduced by approximately 6.3% in patients with MPO/PON1 ratio >3 compared patients with MPO/PON1 ratio <1 (MPO/PON1 <1 group vs. MPO/PON1 >3 group, 25.6 ± 8.4% vs.19.3 ± 5.1%, respectively, $p < 0.05$, Fig. 2).

Table 3
Multiple logistic regression analysis for independent determinants of de novo lesion and ISR occurrence.

	OR	95% CI	*P*-value
A. De novo lesion			
MPO/PON1 ratio >1.59	3.48	1.29–9.40	0.014
Gender	2.65	0.88–8.01	0.083
Age	1.01	0.97–1.05	0.724
Smoking	0.98	0.41–2.35	0.969
Hypertension	1.11	0.36–3.46	0.856
Diabetes mellitus	1.91	0.90–4.05	0.093
Dyslipidemia	0.69	0.27–1.76	0.433
B. ISR			
MPO/PON1 ratio >1.59	6.44	2.15–19.32	0.001
Gender	3.50	1.10–11.1	0.063
Age	1.00	0.96–1.04	0.988
Smoking	0.40	0.15–1.05	0.063
Hypertension	2.89	0.77–10.87	0.116
Diabetes mellitus	1.95	0.89–4.28	0.095
Dyslipidemia	0.86	0.32–2.33	0.764

Abbreviations: ISR, in-stent restenosis; OR, odds ratio; CI, confidence interval; MPO, myeloperoxidase; PON, paraoxonase.

4. Discussion

It has been postulated that MPO acts on LDL and HDL to cause chemical and oxidative modifications of the lipid and protein component [6]. In contrast, PON1 potently protects LDL and HDL from oxidative stress [8]. Thus, it seems reasonable that MPO/PON1 ratio represents the function of lipoproteins. The present study demonstrated that MPO/PON1 ratio was independently associated with both restenosis and recurrent CAD, while lipid profile and other conventional risk factors did not show such relationships. Moreover, the HDL isolated from patients with high MPO/PON1 ratio exhibited attenuated anti-inflammatory properties and impairment of cholesterol efflux capacity. This is the first report that documented a direct correlation between MPO/PON1 ratio and function of HDL.

Two excellent works have previously demonstrated that MPO is a valuable predictor for both new-onset CAD and cardiovascular mortality [14,15]. Both the category-free NRI and IDI, novel methods for evaluating improvement in risk discrimination [13], of MPO/PON1 ratio for CAD risk stratification was greater than that of MPO alone, indicating that combination of PON1 with MPO offers greater improvement in CAD risk discrimination than single application of MPO.

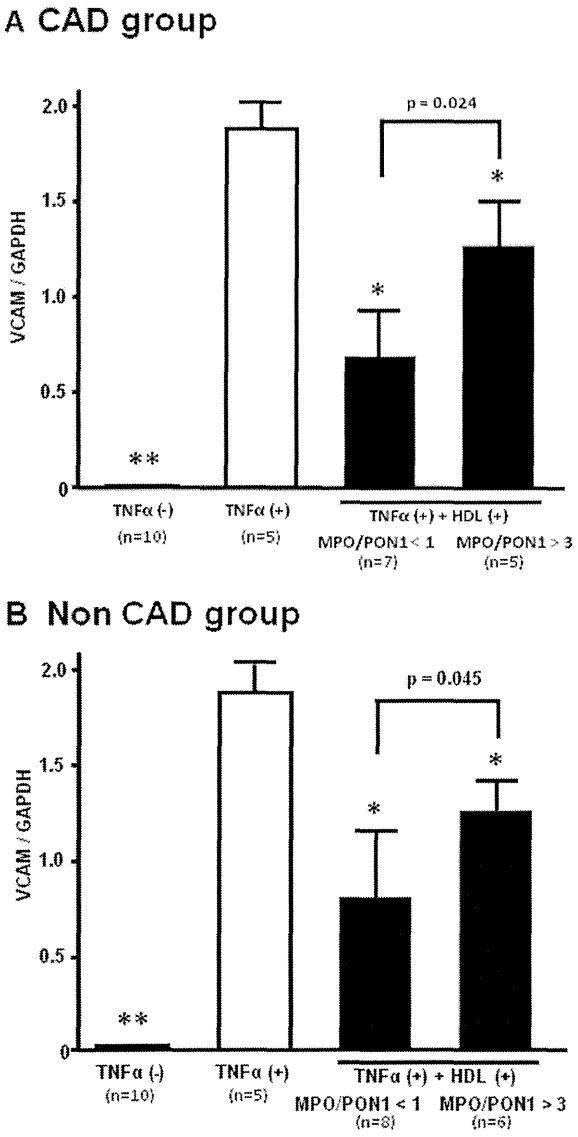

Fig. 1. Inhibition of tumor necrosis factor (TNF)-α induced vascular cell adhesion molecule (VCAM)-1 by high-density lipoprotein (HDL). Human umbilical vein endothelial cells were incubated with or without HDL for 16 h, and then stimulated with or without 10 ng/ml of TNFα for 6 h. HDL was purified from patient's serum with myeloperoxidase/paraoxonase 1 (MPO/PON1) ratio <1 or >3. The expression of VCAM1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained by real-time polymerase chain reaction and the value of VCAM was standardized by GAPDH. (A) Comparisons among CAD patients. (B) Comparisons among non CAD patients. *P* values were obtained using analysis of variance and Scheffe's multiple-comparison *t* test. *$p < 0.05$ and $p < 0.01$ vs. TNFα stimulation without HDL pretreatment.

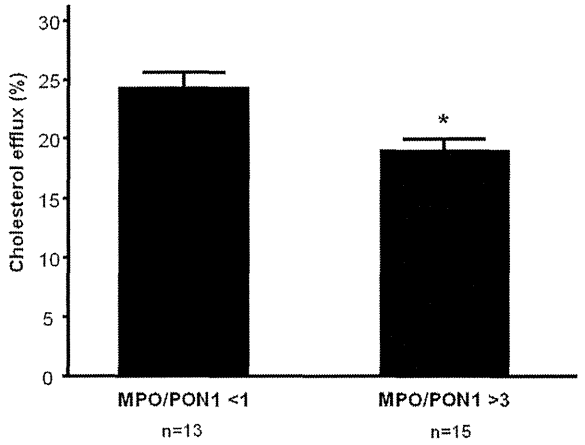

Fig. 2. Effect of MPO/PON1 ratio on HDL efflux capacity. THP-1cells were differentiated into macrophages and loaded with [^{3}H]-cholesterol and TO-901317. After incubation for 4 h with 2% apolipoprotein B-deficient serum, cholesterol efflux was measured ($n = 13$ for patients with MPO/PON1<1 and $n = 15$ for patients with MPO/PON1>3). Values represent mean ± S.D. *Indicates statistically significant differences from patients with MPO/PON1<1 ($P < 0.05$) by Student's *t*-test.

On the other hand, some studies did not document a positive associations between MPO and CAD [16]. The reasons for these contradicting results are speculative, but assay condition in the measurement would have influenced the outcomes. Most previous studies utilized MPO mass instead of its activity, as is the case with this study. Although strong correlations between MPO mass and activity have been reported [16], it remains controversial which reflects the accurate in vivo bioactivity of MPO. Enzymatic activity of MPO depends on local concentration of its co-substrate, hydrogen peroxide (H_2O_2), in vivo [17]. Since MPO activity is measured under saturated amounts of substrate, the ex vivo measurement of serum MPO activity may not always reflect the in vivo activity.

A previous study by Inoue et al. showed that PON1 enzyme activities in diabetic patients were significantly lower than those in controls, while there were no differences in serum PON1 concentrations between patients and controls [18]. Moreover, it has been reported that genetic polymorphism 192Q/R affects paraoxonase activity, but not arylesterase activity [18]. Therefore, we assessed PON1 activity instead of mass in the present study, and demonstrated that both paraoxonase and arylesterase activities were associated with the prevalence of coronary lesions in the CAD group, indicating that the results are independent of the genetic polymorphism.

Although the pathogenesis of atherosclerosis and that of restenosis are presumably different, the high MPO/PON1 ratio was independently correlated with not only occurrence of non-target coronary lesions but also ISR. Growing evidence indicates that oxidative stress plays an important role in the formation of hypertrophic neointima through promoting vascular smooth muscle cell proliferation [19]. Angioplasty induces vascular inflammation [20] and up-regulates NAD(P)H oxidase in the vessel wall, followed by superoxide production that can form H_2O_2 [21]. It has been shown that MPO is enriched in human atherosclerotic lesions [22]. In the presence of H_2O_2 and chloride, MPO generates the potent oxidant hypochlorous acid (HOCl), that can induce endothelial nitric oxide synthase (eNOS) uncoupling, whereby eNOS turns into superoxide-producing enzyme [23]. Thus, there is a possibility that MPO is involved in the development of restenosis via induction of oxidative stress. On the contrary, Demirbag et al. have recently reported that ISR was negatively correlated with paraoxonase and arylesterase activities in patients with bare-metal stents, suggesting that anti-inflammatory and anti-oxidative effects of PON1 might contribute to alleviation of restenosis [24].

Recently, Khera et al. have shown that HDL from CAD patients showed less capacity in promoting cholesterol efflux compared to that from health controls and the efflux capacity of HDL is a potent determinant of cardiovascular events [25]. Meanwhile, Patel et al. demonstrated that HDL has less anti-oxidative capacity in patients with acute coronary syndrome compared with non-CAD or stable CAD patients [26]. Thus, accumulating evidence indicates that dysfunctional HDL could be a novel therapeutic target for CAD. However, conventional methods to assess functional changes in lipoproteins have not been established for clinical use. MPO

generates not only atherogenic LDL but also dysfunctional HDL particles [6]. Recent studies demonstrate that MPO modifies apoA-I and reduces its anti-inflammatory and anti-oxidative properties [7]. Huang et al. have shown that HOCl produced by MPO targets Trp72 of apoA-I and impairs ATP-binding cassette transporter A1-dependent cholesterol transport [27]. In contrast, the decreased PON1 activity is likely further to inhibit the anti-atherosclerotic properties of HDL or apoA-I [28]. These lines of evidence support our notion that high MPO with low PON1 activity could be a substitute marker for dysfunctional HDL.

In the present study, HDL-C levels in CAD group were lower than in non-CAD group. However, there was no significant difference in MPO/PON1ratio between these groups.

Since this was just a comparison between the presence and absence of past CAD history, one plausible explanation is that non-CAD group might have included patients with high risk of CAD and vice versa. On the other hand, MPO/PON1 ratio was significantly different among CAD patients depending on the prevalence of coronary lesion recurrence, while HDL-C levels did not showed such relationship. These findings suggest that quantity of HDL alone is not sufficient for CAD risk stratification, and patients with well-preserved HDL property may have less risk for CAD than those with dysfunctional HDL, even if their HDL-C levels were low.

In conclusion, the present findings suggest that serum MPO mass and PON1 activity as well as MPO/PON1 ratio could be useful markers for CAD risk stratification through modulation of HDL properties.

5. Study limitations

There are several limitations in this study. First, the present study is exploratory because the number of participants was small. In addition, since serum samples were obtained when patients underwent CAG re-examination, the present findings alone are insufficient to draw a conclusion that MPO/PON1 ratio is a predictor of CAD. Therefore, large prospective randomized studies are further required to elucidate the precise role of MPO and PON1 in CAD risk stratification. Second, we measured MPO concentration in serum but not in plasma, while most previous studies assessed plasma MPO concentration. Since PON1 is calcium-dependent, and therefore EDTA sensitive, we had to use serum as a sample material. It should be noted that MPO concentrations in serum are generally higher than in plasma because clot formation activates granulocytes to release MPO [16]. Third, there is a possibility that MPO might promote the progression of atherosclerosis through modulation of LDL function. Recently, Boudjeltia et al. have developed the ELISA system to measure circulating levels of LDL modified by the MPO-H_2O_2-chloride system, commonly named Mox-LDL [29]. This sort of method may provide a better understanding of this issue. In addition, serum PON1 activity dose not entirely reflect HDL-associated PON1 activity alone, although it has been reported that PON1 exhibited higher activity when it binds with HDL than when it is free, and HDL phospholipids affect PON1 catalytic and biological activities [30]. Fourth, it is possible that medications might influence serum MPO/PON1 ratio and/or HDL property. In this study, neither of them showed any correlation with patient's medications including statins (data not shown). However, prospective interventional trial is needed to elucidate this issue as well.

Conflict of interest

This research was supported by Grants-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Japan Foundation of Cardiovascular Research.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2014.03.009.

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Original Article

Predicting Coronary Heart Disease Using Risk Factor Categories for a Japanese Urban Population, and Comparison with the Framingham Risk Score: The Suita Study

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Aim: The Framingham risk score (FRS) is one of the standard tools used to predict the incidence of coronary heart disease (CHD). No previous study has investigated its efficacy for a Japanese population cohort. The purpose of this study was to develop new coronary prediction algorithms for the Japanese population in the manner of the FRS, and to compare them with the original FRS.
Methods: Our coronary prediction algorithms for Japanese were based on a large population-based cohort study (Suita study). The study population comprised 5,521 healthy Japanese. They were followed-up for 11.8 years on average, and 213 cases of CHD were observed. Multiple Cox proportional hazard model by stepwise selection was used to construct the prediction model.
Results: Our coronary prediction algorithms for Japanese patients were based on a large population-based cohort study (the Suita study). A multiple Cox proportional hazard model by stepwise selection was used to construct the prediction model. The C-statistics showed that the new model had better accuracy than the original and recalibrated Framingham scores. The net reclassification improvement (NRI) by the Suita score with the inclusion of CKD was 41.2% ($P<0.001$) compared with the original FRS. The recalibration of the FRS slightly improved the efficiency of the prediction, but it was still worse than the Suita score with the CKD model. The calibration analysis suggested that the original FRS and the recalibrated FRS overestimated the risk of CHD in the Japanese population. The Suita score with CKD more accurately predicted the risk of CHD.
Conclusion: The FRS and recalibrated FRS overestimated the 10-year risk of CHD for the Japanese population. A predictive score including CKD as a coronary risk factor for the Japanese population was more accurate for predicting CHD than the original Framingham risk scores in terms of the C-statics and NRI.

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Key words: Coronary heart disease, Risk score, Japanese cohort, Framingham risk score, Suita study

Introduction

The Framingham Heart Study identified the classic risk factors for coronary heart disease (CHD)[1], and it developed multivariable predictive instruments, which enable clinicians to estimate the 10-year individual risk of developing CHD[2, 3]. These findings have also been widely adopted in clinical guidelines[4, 5]. However, the FRS cannot be generalized for other populations, since 99% of the Framingham cohort participants were Caucasian[5]. For example, the use of the FRS in some other populations resulted in an overestimation of the CHD risk[6-8].

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There has been relatively little attention paid to the validity of the FRS in the Japanese population, which constitutes a unique population in many aspects, with a markedly lower incidence of CHD than Western populations[9]. To our knowledge, no previous Japanese cohort study has been performed to evaluate the original and recalibrated FRS.

Several Japanese cohort studies developed risk prediction tools for Japanese patients. The NIPPON DATA 80 prediction tool has been used as the standard prediction tool in Japan[10], and has been adopted by some clinical guidelines for the stratification of risk in Japanese subjects[11]. However, the NIPPON DATA 80's outcome measure was coronary death, not the incidence of CHD. The Hisayama study predicted a composite outcome of stroke and CHD[12]. Noda's prediction score also applied to cardiac mortality[13]. The JALS study group developed a prediction tool for acute myocardial infarction (AMI), but their prediction period was relatively short (five years)[14]. The JMS-cohort study chart was also targeted for AMI, but the population was limited to rural residents[15]. These tools are all associated with some advantages and disadvantages. However, additional tools for the prediction of CHD are needed that can accurately assess the risk of the longer-term incidence of CHD in the Japanese population.

In this context, we have developed a new algorithm, named the Suita Score, for predicting the 10-year probability of developing CHD, which is based on the findings of a large population-based cohort study performed in an urban area in Japan.

Furthermore, chronic kidney disease (CKD) has recently been advocated as an independent risk factor for CHD, and patients with CKD tend to possess multiple CVD risk factors, and thus represent a major public health problem[16, 17]. A recent CHD risk assessment tool based on 2.3 million patients, the QRISK2, included CKD as a necessary component for the risk prediction[18]. Moreover, CKD patients tend to have an underestimated CHD risk based on the FRS[19]. In addition, we previously reported that CKD leads to an increased risk of both MI and stroke[20]. Hence, the objective of this study was;

1) To incorporate established classic coronary risk factors into newly developed coronary prediction algorithms for the Japanese population,

2) To compare the discriminatory properties of this approach with those of the original and recalibrated FRS

Methods

Populations

The Suita study, a cohort study of urban residents in Japan, was started in 1989. It was based on a random sampling of 12,200 Japanese residents living in Suita. As a baseline, participants between the ages of 30 and 79 years of age were randomly selected from the municipality population registry in 1989. Of these, 6485 males and females underwent regular health checkups between September 1989 and March 1994. The subjects have continued to visit the National Cerebral and Cardiovascular Center (NCVC) every two years for regular health checkups[21-24]. A total of 1,546 subjects were excluded from the study based on a past history of CHD or stroke, non-fasting blood collections, missing data or because they were lost to follow-up. The data from the remaining 5,866 participants (2,788 males and 3,078 females) were used for the analyses. After exclusion of subjects with missing values, total 5,521 (male 2,796 and female 2,725) subjects were used to construct the risk scores. This cohort study was approved by the Institutional Review Board of the National Cerebral and Cardiovascular Center.

Baseline Examinations

Blood samples were collected after the participants had fasted for at least 10 hours. The samples were centrifuged immediately, and a routine blood examination was performed that included the serum total cholesterol (TC), high density lipoprotein cholesterol (HDLC), triglycerides (TG), serum creatinine (Cre) and fasting blood glucose (FBG) levels. The blood pressure was measured three times on the right arm after five minutes of rest by well-trained physicians using a standard mercury sphygmomanometer. The average of the second and third measurements was used for the analyses. Public health nurses obtained information on the smoking and drinking habits and medical histories. To ensure comparability with the FRS, the categorization of the BP, diabetes, TC and HDL-C in this study were done in accordance with the criteria used in the FRS model3. DM was defined as an FBG level ≥ 7.0 mmol/L (126 mg/dl) and/or current use of anti-diabetic medication. Cigarette smoking was dichotomized as current versus non-current. The LDL-C was determined by the Freidewald equation.

Definition of CKD

The serum Cre level was measured using the noncompensated kinetic Jaffe′ method. The estimated

glomerular filtration rate (eGFR) of each participant was calculated from Cre value and the age, using the MDRD equation below, modified with the Japanese coefficient (0.881)[25]:

eGFR (ml/min/1.73 m2) = $0.881*186*age^{-0.203}*Cre^{-1.154}$ (for males)

eGFR = $0.881*186*age^{0.203}*Cre^{-1.154}*0.742$ (for females).

The CKD stage was defined by the K/DOQI clinical practice guidelines[26]. CKD was categorized into Stage 3 CKD (eGFR 30-60 ml/min/1.73m^2) and Stage 4 or 5 CKD (eGFR < 30 ml/min/1.73m^2).

Endpoint Determination

The follow-up method used in the Suita study has been reported previously[20-24]. The endpoints for the current follow-up study were: (1) the date of the first diagnosis of CHD (2) the date of death, (3) the date when the subject left Suita or (4) censoring by December 31, 2007.

The first step in the survey for CHD involved checking the health status of all the participants at clinical visits carried out every two years, and by yearly questionnaires sent by mail or conducted by telephone. The second step involved the review of in-hospital medical records of participants who were suspected to have developed CHD. The criteria for definite or probable acute myocardial infarction were the same as the criteria used for the MONICA project[27].

In order to complete the surveillance for fatal MI, we also conducted a systematic search of death certificates. In addition to acute myocardial infarction, the criteria for a diagnosis of CHD included sudden cardiac death within 24 hours after the onset of acute symptoms, or CHD followed by coronary artery bypass or angioplasty.

Statistical Analysis

First, we evaluated the validity of categorical variables in the Suita Score to compare them with the original FRS[3]. Then, we conducted a multiple Cox proportional hazard model using the same categories as those in the FRS. Subsequently, we developed a new CHD risk score for Japanese subjects based on the Cox model for the Suita cohort. Other risk factors were calculated using the same categories as the FRS. A stepwise selection with a *p*-value of 0.1 for backward elimination was used to select the best predictive model.

After selection of the best Cox model, we fitted the hazard functions developed by the Framingham investigators from the previously published data[6] for predicting the 10-year probability of developing CHD in the Suita cohort. The probability function was: $P = 1 - S(t)$ ^exp(X, M); $f(X, M) = \beta_1*(X_1 - M_1) + \ldots + \beta_n*(X_n - M_n)$,

where S(t) is the survival rate for the mean values of the risk factors at 10 years in the Suita study; $\beta_1 \ldots \beta_n$ are the regression coefficients of the Cox model (β) shown in **Table 3** ; $X_1 \ldots X_n$ represent the individual risk factor values of each study participant and $M_1 \ldots M_n$ are the mean values of the risk factors in the Suita cohort. In the recalibrated Framingham functions, the coefficients were taken from the Framingham Cox model, but the mean values from the Suita cohort were used for the risk factors and the mean incidence rates[6].

Discrimination and calibration were used to evaluate the predictive capabilities of the models. We evaluated the discriminatory ability of this model by comparing the means of the C-statistics and Bayesian information criteria (BIC). Furthermore, we measured the model improvement as indicated by the clinical reclassification of the FRS by the Suita Score, which is considered to be more important indicator for predictive ability using the net reclassification improvement (NRI)[28]. Since the inclusion of a new biomarker in a prediction tool , such as the FRS, minimally improves the predictive ability, the evaluation based on the NRI is considered to be a valid approach for evaluating the new biomarker[29]. The NRI measures the reclassification of people from one risk category to another resulting from the addition of the new risk factor to a prediction model with established risk. If all of the people end up in a more correct risk class based on the model with the new marker, the NRI is positive. We calculated the category-free NRI[30].

The third approach was calibration, which measured how closely the predicted risk fit the actual risk. The Suita participants were divided into quintiles of 10-year CHD risk predicted by the Suita score functions, the original Framingham functions and the recalibrated Framingham functions[6]. The predicted and actual risk in each quintile were compared, and the differences were assessed by the Hosmer-Lemeshow chi-square tests. The SAS software program, version 9.3. (SAS Institute Inc), and the STATA software program, version 12 (STATA Corp LP), were used for all of the statistical analyses.

Results

Population Characteristics

The number of person-years studied consisted of 75,776 (34,480 for males and 41,296 for females), with a mean follow-up period of 11.8 years. During

Table 1. Population characteristics of the study cohort

	Males (n=2796)	Females (n=2725)
Age (years, mean ± SD)	56.1 ± 13.3	54.5 ± 12.9
DM (%)	6	5.8
Current smoker (%)	49.67	11.91
Blood pressure (mmHg, %)		
Optimal (SBP < 120, DBP < 80)	30.74	41.68
Normal (SBP < 130, DBP < 85)	19.31	17.30
High normal (SBP < 140, DBP < 90)	17.98	15.69
Stage I HT (SBP < 160, DBP < 100)	20.39	15.94
Stage II to IV HT (SBP > = 160, DBP ≥ 100)	11.59	9.40
Total cholesterol (mg/dl, %)		
< 160	10.12	6.88
160-199	39.75	30.52
200-239	37.41	39.60
240-279	10.98	18.51
≥ 280	1.74	4.49
LDL cholesterol (mg/dl, %)		
> 130	55.54	45.78
130-159	28.19	30.86
> 160	16.26	23.34
HDL cholesterol (mg/dl, %)		
< 35	11.40	3.28
35-44	28.71	16.36
45-49	15.87	12.25
50-59	23.82	29.95
≥ 60	20.20	38.14
Creatinine (mg/dl, mean ± SD)	0.91 ± 0.21	0.69 ± 0.22
eGFR (mean ± SD)	64.7 ± 24.9	90.6 ± 29.3
CKD (≥ Stage 3) (%)	46.2	11.3

LDL, Low-density lipoprotein; HDL, high-density lipoprotein;
eGFR, estimated glomerular filtration rate (ml/min/1.73 m^2); CKD, chronic kidney disease; HT, hypertension

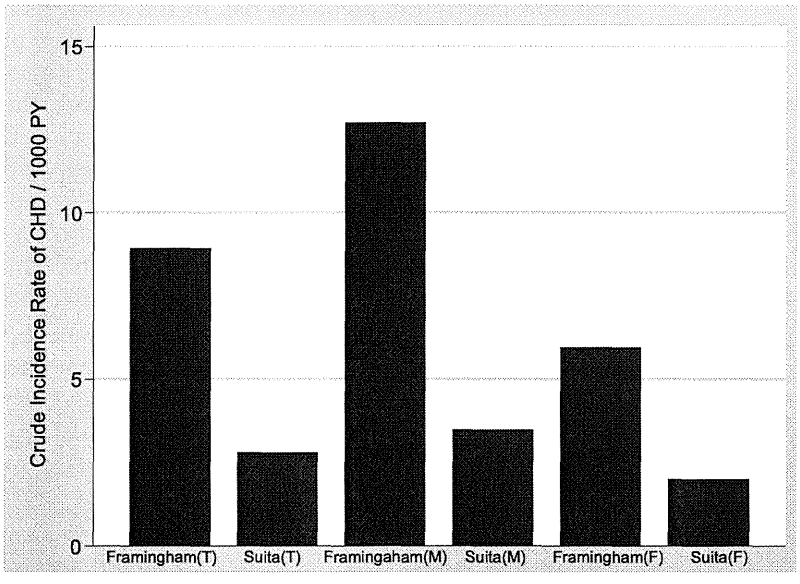

Fig. 1. The absolute risk difference of the Framingham cohort and Suita Study cohort

The Framingham cohort data were adopted from Wilson's study
CHD, coronary heart disease; PY, person-years; Framingham(T), total Framingham cohort; Framingham(M), Male Framingham cohort; Framingham(F), Female Framingham cohort; Suita(T), total Suita cohort; Suita (M), Male Suita cohort; Female Suita (F), Suita cohort

Table 2. The multivariable-adjusted hazard ratios for coronary heart disease based on the FRS categories

MALES				
Variable	Relative Risk	*P*-value	95% CI	Framingham Cohort
Age, y	1.07	<0.001	1.05-1.09	1.05
TC, mg/dl				
<200	Reference			
200-239	1.30	0.172	0.89-1.88	1.31
≥ 240	2.15	0.001	1.38-3.34	1.9
HDL-C mg/dl				
<35	2.06	0.001	1.37-3.10	1.47
35-59	Reference			
≥ 60	0.67	0.103	0.42-1.08	0.56
Blood Pressure				
Normal (including optimal)	Reference			
High normal	1.52	0.104	0.92-2.51	1.31
Stage I hypertension	2.24	<0.001	1.45-3.46	1.67
Stage II hypertension	2.34	0.001	1.41-3.86	1.84
Diabetes (y/n)	1.39	0.234	0.81-2.40	1.5
Smoking	1.25	0.193	0.89-1.76	1.68
CKD	1.34	0.109	0.94-1.92	N.A
FEMALES				
Variable	Relative Risk	*P*-value	95% CI	Framingham Cohort
Age, y	1.10	<0.001	1.07-1.13	1.04
TC, mg/dl				
<200	Reference			
200-239	0.58	0.097	0.30-1.10	1.51
≥ 240	1.38	0.272	0.78-2.46	1.72
HDL-C mg/dl				
<35	1.94	0.102	0.88-4.31	2.02
35-59	Reference			
≥ 60	1.04	0.881	0.61-1.79	0.58
Blood Pressure				
Normal (including optimal)	Reference			
High normal	1.60	0.222	0.75-3.38	1.3
Stage I hypertension	1.82	0.089	0.91-3.61	1.73
Stage II hypertension	3.86	<0.001	1.99-7.48	2.12
Diabetes (y/n)	2.59	0.013	1.23-5.49	1.77
Smoking	3.22	<0.001	1.74-5.97	1.47
CKD	1.38	0.247	0.80-2.40	N.A

FRS, Framingham risk score; All variables were adjusted for all FRS variables by a Cox proportional hazard model. CKD, chronic kidney disease; 95% CI, 95% confidence interval; N.A, not available

the follow-up period, there were 213 incidents of CHD. The population characteristics are summarized in **Table 1**. The univariate Cox regression analysis indicated all variables in FRS were statistically significant (data not shown).

Fig. 1 depicts the difference in the absolute risk for CHD between the Framingham cohort and Suita study cohort. The crude incidence rate of CHD in the original Framingham cohort was 8.94 per 1000 person-years, while that of the Suita cohort was 2.81 per 1000 person-years. The risk of developing CHD is nearly one-third of both the males and females in this study cohort.

The results of the multiple Cox proportional

hazard model using the same categories as those used in the FRS are shown in **Table 2**. All hazard ratios (HRs) of categorical hypertension were higher than those of the original FRS. The HRs of smoking and DM for females were also higher than those of the original FRS (2.59 and 3.22, respectively). The HR of a TC between 200 and 239 for females was 0.58, which was lower than that of the FRS. The HRs of other variables were similar to those of the original FRS.

Prediction Model Development and the Simplified Prediction Model for Clinical Use

Table 3 shows the best Cox model for the Suita cohort selected by a stepwise method with the total cholesterol categories. (TC Suita score) The multivariable adjusted HR for the association between CHD and Stage 3 CKD was 1.39 and that for Stage 4 and 5 CKD was 3.72, respectively. The HRs of the other predictors were similar between the with CKD and without CKD models.

Table 4 shows the best Cox model for the Suita Score with CKD according to the cut-off levels of LDL-C and HDL-C proposed in the Japan Atherosclerosis Society (JAS) Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases 2012[11, 31] (LDL Suita Score). For convenient clinical use, we developed prediction sheets based on the TC and LDL Suita Scores (**Table 5**). The beta coefficients corresponding to the Cox model were multiplied 10 times for categorical covariates and were rounded. For the age category, the midpoint of each category was multiplied by the β coefficients in **Table 4**, and then multiplied 10 times. We added all these values corresponding to each individual risk, divided the number by 10, and then the corresponding probability of CHD was calculated from the equation: P=1−S(t)^exp((sum of the points)/10) where S(t) is the baseline survival function of the Suita cohort.

The C-statistics of the LDL Suita Score with CKD in **Table 4**, which corresponded to the AUC of the Cox proportional hazard model, was 0.831. This was very similar to the TC Suita Score shown in **Table 3**, which had a C-index of 0.835 (**Table 6a**). The likelihood ratio test was not conducted, since the categorical variables were different and these two models were not nested. The NRI between TC Suita Score with CKD and the LDL Suita Score with CKD was not significant (P=.0.256; **Table 6b**). These findings suggest that the two models predict CHD with similar efficiency.

Validation of the Inclusion of CKD

The C-static of the Suita Score without CKD was slightly lower than the Suita Score with CKD (0.835 vs. 0.833). The comparison between the TC Suita Scores with and without CKD suggested that the addition of CKD improved the risk classification of CHD by 40%. This suggested that the inclusion of CKD in the risk prediction tool improves the prediction of the development of CHD, making it a more appropriate predictive tool.

Comparison of the Suita Score and Framingham Risk Scores

Table 7a shows the model fit, C-statistics and BIC of the Cox regression for the TC Suita Score, the original FRS and the recalibrated score for the mean value of each of the covariates. The TC Suita Score with CKD showed the best goodness-of-fit by the likelihood ratio test, and the C-statistics of the TC Suita Score with CKD were also the highest. The BIC was the lowest for the TC Suita Score with CKD, which supported its better predictive ability. The C-statistics were not changed by the recalibration of the FRS. The C-statistics of the recalibrated FRS were still smaller than the TC Suita Score with CKD.

The results of the clinical reclassification measured by the NRI are shown in **Table 7b**. The NRI for the TC Suita Score with CKD compared to the original FRS was 46.8% (P<0.001). In both the CHD and non-CHD groups, the risk categories tended to be increased by the TC Suita Score with CKD. The NRI between the TC Suita Score with CKD and the recalibrated model was lower (25.4%), but the difference remained significant (P=0.003). These associations also held for the TC Suita Score without CKD, the FRS and the recalibrated FRS.

Fig. 2 depicts the actual and predicted probabilities of the 10-year risk of cardiac events by calibration. The FRS consistently overestimated the cardiac events in all quintiles. The overall 10-year calibration of the FRS and recalibrated FRS were worse than the TC Suita Score with CKD as determined by the Hosmer-Lemeshow chi-square test (both p<0.001). The largest difference between the actual rate and the predicted rate after recalibration was 13.9% (in the fifth quintile in males), compared with the difference of 14.5% for the FRS. The difference between the actual probability and the TC Suita Score with CKD was not significant (P=0.18). The TC Suita Score with CKD model underestimated the risk of CHD in the fourth quintile, but the difference was only 2.2%. These findings consistently indicated that the FRS overestimates the CHD risk in the Japanese population.